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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 0274-3858.1US (U-2429)

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 10/048244

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/40495	27 July 2000 (27.07.2000)	27 July 1999 (27.07.1999)

TITLE OF INVENTION
HOMOGENEOUS FLUORESCENCE METHOD FOR ASSAYING STRUCTURAL MODIFICATIONS OF BIOMOLECULES

APPLICANT(S) FOR DO/EO/US
Donald K. Blumenthal

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. [X] The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is attached hereto (required only if not communicated by the International Bureau).
   b. [ ] has been communicated by the International Bureau.
   c. [X] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [ ] is attached hereto.
   b. [ ] has been previously submitted under 35 U.S.C. 154(d)(4).
7. [X] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A FIRST preliminary amendment.
14. [ ] A SECOND or SUBSEQUENT preliminary amendment.
15. [ ] A substitute specification.
16. [ ] A change of power of attorney and/or address letter.
17. [ ] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. [ ] Other items or information:

NOTICE OF EXPRESS MAILING
Express Mail Mailing Label Number: EL740546751US
Date of Deposit with USPS: January 25, 2002
Person making Deposit: Teresa G. Nielsen

U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
10/048244	PCT/US00/40495	0274-3858.1US

21. ☒ The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1000.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $860.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $710.00		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) $690.00		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 690.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	27 - 20 =	7	x $18.00	$	126.00
Independent claims	6 - 3 =	3	x $80.00	$	240.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $270.00	$	
TOTAL OF ABOVE CALCULATIONS =				$	1,186.00
☒ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. +				$	593.00
SUBTOTAL =				$	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				$	
TOTAL FEES ENCLOSED =				$	593.00
				Amount to be refunded:	$
				charged:	$

a. ☒ A check in the amount of $ 593.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 20-1469 . A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO.

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SIGNATURE

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30,549
REGISTRATION NUMBER

HOMOGENEOUS FLUORESCENCE METHOD FOR ASSAYING STRUCTURAL MODIFICATIONS OF BIOMOLECULES

PRIORITY CLAIM

This application claims the benefit of the filing date of United States Provisional Patent Application Serial Number 60/145,755, filed July 27, 1999, for "HOMOGENEOUS FLUORESCENCE METHOD FOR ASSAYING STRUCTURAL MODIFICATIONS OF BIOMOLECULES".

TECHNICAL FIELD

The invention relates to methods useful for the homogenous assay of covalent modifications to biomolecules which utilize double-labeled biomolecular substrates. Also described are methods of preparing and characterizing the double-labeled biomolecular substrates and methods of using the inventive assay for high-throughput screening, for diagnostic and therapeutic applications, and for discovering substrates of novel enzymes.

BACKGROUND

The healthy development and function of eukaryotic organisms depends upon the proper regulation of structural modifications of various biomolecules (the phrases "covalent modification" and "structural modification" are used interchangeably herein). It is believed that virtually all intracellular biochemical processes in eukaryotes are regulated in some fashion by the covalent modification of biomolecules, such as proteins or peptides. However, where an intracellular process responsible for the covalent modification of a particular type of biomolecule is somehow dysfunctional, several disease states can result.

For example, protein kinases represent one of the largest superfamilies of enzymes in eukaryotic organisms, with an estimated 1-3% of the human genome coding for various protein kinases. Protein kinases catalyze the transfer of phosphate from ATP to specific amino acids in proteins, and phosphorylation of proteins is known to be the most widespread mechanism for reversible covalent modification of protein structure and function. The dysfunctional regulation of protein phosphorylation is

believed to result in several diseases, such as, for example, diabetes, cancer, and many forms of heart disease.

As can be appreciated, the ability to assay the activity of the various intracellular processes responsible for the covalent modification of particular biomolecules is essential in order to gain an understanding of the potential roles such processes play in normal cells and various disease states. Assay techniques which detect and quantify various types of covalent modifications of particular biomolecules would also facilitate the development of diagnostic and therapeutic technologies relating to disease states resulting from dysfunctional modification processes. An assay technique ideally suited for these purposes would be sensitive and continuous, would allow both *in vitro* and *in vivo* assays, would be efficient and economical, and would enable high-density, high-throughput screening.

A variety of methods are currently used to assay the covalent modification of biomolecules. Most of these methods, however, are relatively inefficient or uneconomical in that they require the use of radioactive labels, multi-component assay systems, and/or multi-step procedures. Moreover, most existing methods of assaying the structural modification of biomolecules are discontinuous and, therefore, necessitate the sampling of the reaction at specific times in order to determine enzymatic activity.

Unlike most known methods, U.S. Patents 5,776,720 to Jaffe et al. (July 7, 1998), 5,770,691 to Fields et al. (June 23, 1998), 5,763,181 to Han et al. (June 9, 1998), 5,733,719 to Jaffe et al. (March 31, 1998), and 5,698,411 to Lucas et al. (December 16, 1997) teach various methods for assaying enzyme-mediated cleavage of biomolecules, such as peptides or nucleic acids, which do not require radioactive labels and which are continuous and homogenous. However, the usefulness of even these methods is limited to the assay of enzyme-mediated cleavage reactions, and such reactions constitute only one subset of the many processes by which biomolecules are structurally modified within the cell.

Homogenous assay methods which detect the presence of antibodies, nucleic acids, or protein kinase activity through the use of fluorogenic tracer molecules are also known. For example, PCT International Publication No. WO/03429 ("the WO/03429 publication") teaches homogenous assay techniques which utilize fluorogenic tracer molecules that exhibit a change in fluorescence upon association with their target

antibody or nucleic acid sequence. Though the WO/03429 publication provides no solution to the challenge of developing methods or compositions for monitoring or detecting covalent biomolecular modifications, a protein kinase assay technique making use of the technology disclosed in the WO/03429 application was recently reported in an article authored by Geoghegan et al. ("Geoghegan et al.") (*See,* Geoghegan et al., *Bioconjugate Chemistry*, 11:71-77 (2000)).

The assay technique disclosed in Geoghegan et al. is relatively complicated and expensive, however, and the technique unsuitable for *in vivo* application. The assay technique disclosed in Geoghegan et al. uses double-labeled tyrosine kinase substrate peptides and a polypeptide corresponding to an SH2 domain. The SH2 domain binds the double-labeled tyrosine kinase substrate peptides when those substrate peptides are phosphorylated, and binding of the SH2 domain to the phosphorylated double-labeled tyrosine kinase substrate peptides causes the two labels included in each of the substrate peptides to dissociate, resulting in a change in fluorescence. Thus, the assay technique disclosed in Geoghegan et al. requires the interaction of two expensive components (*i.e.*, the interaction of double-labeled tyrosine kinase substrate peptides with SH2 domain peptides), resulting in a technique that is relatively complex and expensive. Moreover, due to its use of a multi-component system, the assay technique of Geoghegan et al. is not suitable for performing *in vivo* assays.

Homogenous fluorescent protein kinase assay methods utilizing various Green Fluorescent Protein ("GFP") molecules have also been recently reported. For instance, an article written by Nagai et al. teaches a method for assaying protein kinase activity using a Kinase-Inducible Domain construct containing two GFP groups (*See*, Nagai et al., *Nature Biotechnology*, 18:313-316 (2000)). The assay method taught by Nagai et al., however, depends upon phosphorylation-dependent changes in the fluorescence resonance energy transfer ("FRET") among the two GFP groups to detect kinase activity, and because such changes in FRET are small, the assay technique of Nagai et al. does not provide a homogenous assay having a desired level of sensitivity. In addition, U.S. Patent 5,912,137 ("the '137 Patent") teaches a protein kinase assay utilizing modified GFP molecules as assay substrates. However, because the assays taught in the '137 patent can only be carried out using modified GFP substrates, the potential applications of such assays are limited.

Because of the deficiencies existing in the known assay methods, it would be an improvement in the art to develop a homogenous assay method which not only enables the continuous, real-time assay of covalent biomolecular modifications, such as phosphorylation, sulfation, glycation, glycosylation, carboxylation, myristoylation, farnesylation, ubiquitination, and biotinylation, but which also provides sensitive and economical assays that are simple to carry out *in vitro* and *in vivo* and are adaptable to a wide variety of applications.

DISCLOSURE OF INVENTION

The present invention includes substrates and methods useful for the assay of covalent modification of biomolecules. In contrast to assay methods already in use, due to the nature of the double-labeled molecular substrates described herein, the assay methods of the present invention are sensitive and homogeneous and do not require the use of radioisotopes. The assay methods herein disclosed are also relatively simple and economical, adaptable to a wide variety of applications, easily used *in vitro* and in living cells, and allow continuous, real-time monitoring of structural modifications to biomolecules. Moreover, the assay methods of the present invention are useful for detecting and quantifying a wide range of covalent biomolecular modifications which do not result in the cleavage of the biomolecule. Thus, the present invention offers significant advantages in terms of simplicity, efficiency, and scope when compared to presently used methods for detecting covalent biomolecular modifications.

The substrates of the present invention are double-labeled biomolecular substrates (the phrases "double-labeled biomolecular substrate" and "double-labeled substrate" are used interchangeably herein). The double-labeled substrates of the present invention include a core molecular backbone covalently labeled at two positions with a first fluorescent dye and a second fluorescent dye or with a first fluorescent dye and a second non-fluorescent dye (for convenience, the term "dye" is used herein to describe a chromophoric or fluorophoric moiety). The core molecular backbone of a double-labeled substrate according to the present invention may include a protein or peptide sequence, a nucleotide sequence, a sugar, a lipid, a receptor, or a biopolymer. As used in the context of the present invention, the term "biopolymer" includes any molecule that is a covalent combination of amino acids, nucleic acids, sugars, lipids, or

other small molecules of biological origin. The core molecular backbone may also include a substrate determinant specific for a particular process of covalent biomolecular modification. Furthermore, each double-labeled substrate of the present invention is constructed and labeled so that when the double-labeled substrate is in its unmodified state, the first and second labeling dyes associate, or stack, to form an intramolecular dimer. When the first and second labeling dyes form an intramolecular dimer, the fluorescence of one or both of the dyes is quenched, thereby quenching the fluorescence of the double-labeled substrate. Upon catalytic or non-catalytic covalent modification of the double-labeled substrate, however, the first and second labeling dyes dissociate, and the fluorescence of the covalently modified double-labeled substrate changes markedly. Therefore, because only the modified double-labeled substrate exhibits a change in fluorescence, no need exists to separate the modified double-labeled substrate from the unmodified double-labeled substrate in order to accurately assay the extent to which an amount of double-labeled substrate has been covalently modified. As a result, the invention enables homogenous and continuous assay methods which are simple and economical, and which may be employed both *in vitro* and in living cells.

It should be noted that the quenching phenomenon underlying the substrates and fluorescence assay methods of the present invention is not fluorescence resonance energy transfer ("FRET"). Unlike FRET, the quenching phenomenon underlying the substrates and fluorescence assay methods of the present invention involve ground state interaction of two dyes. Advantageously, because the quenching phenomenon described herein involves ground state interactions which result in changes in the absorbance spectra of the two dyes included in a double-labeled substrate according to the present invention, double-labeled substrates of the present invention may be used for homogenous absorbance-based assays detecting various types of structural modifications of biomolecules through modification-dependent changes in the absorbance spectra of the double-labeled substrates.

The assay methods of the present invention are versatile. Because the core molecular backbone of double-labeled substrates of the present invention can be constructed to include substrate determinants for a wide range of intracellular processes, the assay methods of the present invention are applicable to a broad range of

covalent biomolecular modifications. For example, the core molecular backbone of a double-labeled substrate may include a protein kinase substrate determinant. Such a double-labeled substrate could then be used to assay the activity of a particular protein kinase or a particular class of protein kinases. However, this is but one example. Double-labeled substrates of the present invention could be constructed in order to assay numerous other modification reactions, such as sulfation, glycation, glycosylation, carboxylation, myristoylation, farnesylation, ubiquitination, and biotinylation, by which biomolecules are structurally modified.

Various other methods are also included within the scope of the present invention. For example, methods of producing the assays of the present invention and of producing and characterizing the double-labeled biomolecular substrates of the present invention are described herein. Also described herein are methods for using the inventive assay procedure in high-throughput screening, methods for monitoring the activity of the intracellular processes by which biomolecules are covalently modified, methods for diagnostic and therapeutic applications of the inventive substrates and assay procedures, and methods for discovering substrates for novel enzymes involved in the covalent modification of particular biomolecules.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 provides a schematic illustration of a first embodiment of a double-labeled biomolecular substrate of the present invention in an unmodified state.

FIG. 2 is a schematic illustration of a first embodiment of a double-labeled biomolecular substrate of the present invention in a structurally modified state.

FIG. 3 schematically depicts the modification-dependent transition between intramolecular dimer and intramolecular monomer states of a double-labeled biomolecular substrate according to the present invention.

FIG. 4 is a schematic illustration of a second embodiment of a double-labeled biomolecular substrate of the present invention in a structurally modified state.

FIG. 5 illustrates the absorbance spectrum of double-labeled substrate including a fluorescein and a rhodamine label before and after phosphorylation by PKA.

FIG. 6 illustrates the fluorescent emission of the fluorescein label of the double-labeled substrate of FIG. 5 before and after phosphorylation by PKA.

FIG. 7 illustrates the fluorescent emission of the rhodamine label of the double-labeled substrate of FIG. 5 before and after phosphorylation by PKA.

FIG. 8 illustrates an absorbance spectrum of double-labeled substrate including two rhodamine labels before and after phosphorylation by PKA.

FIG. 9 is a chart of the fluorescence the rhodamine labels of the double-labeled substrate of FIG. 8 before and after phosphorylation by PKA.

FIG. 10. schematically illustrates a double-labeled substrate including a covalently attached enhancer, the double-labeled substrate transitioning between an unphosphorylated and a phosphorylated state upon phosphorylation by PKA.

BEST MODES FOR CARRYING OUT THE INVENTION

A first embodiment of a double-labeled biomolecular substrate according to the present invention is schematically illustrated in its unmodified state in FIG. 1. The double-labeled substrate 10 includes a core molecular backbone 20 containing a substrate determinant 30 which facilitates the modification of the double-labeled substrate, a fluorescent dye 40, and a quenching dye 50. As is illustrated in FIG. 1, the double-labeled substrate 10 is constructed so that, in the substrate's unmodified state, the fluorescent dye 40 and the quenching dye 50 associate, or stack, to form an intramolecular dye dimer 55. The formation of an intramolecular dye dimer 55 results in the quenching of the fluorescent dye 40 by the quenching dye 50. Thus, in its unmodified state, the fluorescence of the double-labeled substrate 10 is markedly reduced.

FIG. 2 provides a schematic illustration of the double-labeled substrate 10 of FIG. 1 after the double-labeled substrate 10 has been structurally modified by, for example, a protein kinase. As is illustrated in FIG. 2, the protein kinase adds a phosphate group 60 to an amino acid residue of the substrate determinant 30 included in the core molecular backbone 20 of the double-labeled substrate 10 (protein kinases most commonly phosphorylate hydroxyl amino acids, such as serine, threonine, and tyrosine). Phosphorylation of the double-labeled substrate 10 results in the dissociation of the fluorescent dye 40 and the quenching dye 50 and, thus, the dissociation of the intramolecular dye dimer 55, which, in turn, results in a marked increase in the fluorescence of the double-labeled substrate because the quenching dye 50 no longer

quenches the fluorescence of the fluorescent dye 40. The double-labeled substrate 10 only exhibits a change in fluorescence upon covalent modification, in this case phosphorylation. Thus, the extent to which an amount of double-labeled substrate of the present invention is structurally modified can be continually assayed without separation steps merely by quantifying various changes in substrate fluorescence.

FIG. 3 schematically illustrates the modification dependent transition between intramolecular dimer and intramolecular monomer of the two dyes 80, 90 of a double-labeled substrate 70 of the present invention using a peptide based on the Kinase-Inducible Domain (KID) of the Cyclic AMP Response Element Binding Protein (CREB) and Protein Kinase A ("PKA") as an illustrative system. CREB is a well-known substrate for PKA and other protein kinases, and FIG. 3 illustrates a double-labeled substrate 70 of the present invention which includes the KID sequence of CREB within the core molecular backbone 75, and two dyes 80, 90 conjugated to the KID sequence. In the double-labeled substrate's 70 unphosphorylated state, the two dyes 80, 90 form an intramolecular dye dimer 95 and the fluorescence of the fluorescent dye 80 is quenched. When the double-labeled substrate is reacted with PKA however, a phosphate group, or "P" 100, is introduced in the double-labeled substrate 70, and the intramolecular dye dimer 95 dissociates, resulting in an increase in the fluorescence of the flourescent dye 80.

Though the double-labeled substrate of the present invention and the modification-dependent transition of double-labeled substrates of the present invention have thus far been described in the context of protein kinase activity, such descriptions are illustrative only and do not limit the scope of the present invention. The double-labeled substrates of the present invention are useful for assaying a broad range of structural modifications to various biomolecules and can be specifically constructed for the assay of numerous processes of covalent biomolecular modification. The core molecular backbone of double-labeled substrates of the present invention may be constructed to include protein or peptide sequences, nucleotide sequences, sugars, lipids, receptor molecules, biopolymers, or virtually any other biomolecule which may serve as a substrate in one or more intracellular processes of covalent biomolecular modification. Thus, double-labeled substrates of the present invention can be constructed for the assay of numerous catalytic and non-catalytic processes of covalent

biomolecular modification. By way of example, double-labeled substrates according to the present invention could be constructed to exhibit a change in fluorescence upon sulfation, glycation, glycosylation, carboxylation, myristoylation, farnesylation, ubiquitination, biotinylation, or other modification reactions.

Although not intending to be bound by a particular theory of the invention, the following explanations might explain the excellent results of the invention. For instance, the dissociation of intramolecular dye dimer formed by the two dyes could be the result of one or more different mechanisms. The simplest mechanism of intramolecular dye dimer dissociation would be through steric and/or electrostatic effects resulting from the introduction of a functional group into the double-labeled substrate in close proximity to the intramolecular dye dimer. Another possible mechanism for the dissociation of the intramolecular dye dimer is through a modification-dependent conformational change in the double-labeled substrate. Yet another possible mechanism for the dissociation of the intramolecular dye dimer is through a conformational change in the double-labeled substrate brought about by the modification-dependent binding of a second molecule to the double-labeled substrate. These second molecules could be considered as fluorescence enhancers. For example, where the double-labeled substrate is modified by the addition of a phosphate group to a tyrosyl residue in a peptide substrate, the enhancer molecule might be a phosphotyrosine-specific antibody or a SH2-domain-containing protein with a high affinity for phosphotyrosine. As is illustrated in FIG. 10, an enhancer molecule 200 could be covalently combined with the double-labeled substrate 10 to form a single chimeric molecule.

A second embodiment of the double-labeled substrate of the present invention is schematically illustrated in FIG. 4. The double-labeled substrate 110 of FIG. 4 is illustrated in its modified state for ease of description and includes a core molecular backbone 120, a substrate determinant 125 within the core molecular backbone 120, a first spacer segment 130 and a second spacer segment 140. The first spacer segment 130 is included at a first terminus 135 of the core molecular backbone 120, and the second spacer segment 140 is included at a second terminus 145 of the core molecular backbone 120. The first or second spacer segments 130, 140 may be included in the double-labeled substrate 110 to provide a region of flexibility between

the core molecular backbone 120 and one or both of the dyes 150, 160 also included in the double-labeled substrate 110. By providing a region or regions of flexibility between the core molecular backbone 120 and one or both the dyes 150, 160, the spacer segments 130, 140 may ease the formation of an intramolecular dye dimer, and thus facilitate more complete quenching of double-labeled substrate 110, resulting in a more sensitive assay. The first or second spacer segments 130, 140 may also be included in the double-labeled substrate 110 in order to facilitate the use of a particular dye which cannot be conjugated to one of the amino acid residues included in the core peptide sequence 120.

The embodiment illustrated in FIG. 4 highlights that the exact construction of double- labeled biomolecular substrates of this invention will vary. The construction of a double-labeled substrate may vary not only by including one or more spacer segments which may facilitate a more sensitive assay or enable the use of different dyes, but the construction may vary to utilize biomolecular substrates corresponding to different processes of covalent biomolecular modification. For example, within the family of protein kinases, double-labeled substrates of the present invention could be constructed using core molecular backbones which include, among others, the following amino acid sequences: Arg-Arg-Arg-Val-Thr-Ser-Ala-Ala-Arg-Arg-Ser (SEQ. ID. NO.: 9), a substrate peptide for Protein Kinase A and Protein Kinase C (*See, e.g.*, PCT International Application WO Patent Document 98/09169); Phe-Arg-Arg-Leu-Ser-Ile-Ser-Thr (SEQ. ID. NO.: 1) and Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ser-Ser (SEQ. ID. NO.: 2), substrate peptides for Ca^{2+}/calmodulin-dependent protein kinase II (*See, e.g.*, PCT International Application WO Patent Document 98/09169; Pearson et al., *Journal of Biological Chemistry*, 260(27), 14471-76 (1985)); Phe-Leu-Thr-Glu-Tyr-Val-Ala-Thr-Arg-Trp-Tyr-Arg-Ala-Pro-Glu (SEQ. ID. NO.: 3), a substrate peptide for mitogen-activated protein kinase kinase (*See*, Rossomondo et al., *Proceedings of the National Academy of Science USA*, 89, 5221-25 (June 1992)); or Arg-Arg-Asp-Ile-Tyr-Glu-Thr-Asp-Tyr-Tyr-Arg-Lys (SEQ. ID. NO.: 4), a substrate peptide for insulin receptor protein-tyrosine kinase (*See*, Dickens et al., *Biochemical and Biophysical Research Communications*, 174(2), 772-84 (1991)). These examples, however, illustrate only a few of the potential substrate determinants which may be included in double-labeled substrates of the present invention. These examples do not reflect the myriad of other

the core molecular backbone 120 and one or both of the dyes 150, 160 also included in the double-labeled substrate 110. By providing a region or regions of flexibility between the core molecular backbone 120 and one or both the dyes 150, 160, the spacer segments 130, 140 may ease the formation of an intramolecular dye dimer, and thus facilitate more complete quenching of double-labeled substrate 110, resulting in a more sensitive assay. The first or second spacer segments 130, 140 may also be included in the double-labeled substrate 110 in order to facilitate the use of a particular dye which cannot be conjugated to one of the amino acid residues included in the core peptide sequence 120.

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biomolecular substrates which may be included in the core molecular backbone of double-labeled substrates of the present invention. Again, the double-labeled substrates of the present invention have broad application and can be tailor-made for use with any one of many enzymatically catalyzed or non-catalyzed intracellular processes by which biomolecules are covalently modified.

It is also possible to create double-labeled substrates according to the present invention using a variety of dyes and combinations of dyes. For example, the dyes may be conventional fluorescent dyes, such as fluorescein, rhodamine, cyanine, Oregon Green, Texas Red, Lucifer Yellow, BODIPY, rhodol, coumarin, pyrene, eosin, erythrosin, napthalene, pyridyloxazole, anthrancene, fluorescamine, acridine, benzofuran, anthranilic acid, aminobenzoic acid, N-methylisatoic acid, isoluminol, bezoxadiazole, carboxybenzoyl-quinoline-carboxyaldehyde, salicylate, bimane, or phenathroline, or the dyes may be non-conventional fluorescent dyes, such as a Yellow Fluorescent Protein (YFP) or a Green Fluorescent Protein (GFP) (obtainable from CLONTECH Laboratories, 1020 East Meadow Circle, Palo Alto, CA 94303). In addition, the publication entitled "*Handbook of Fluorescent Probes and Research Chemicals*," by Richard P. Haugland, which serves as a catalog for Molecular Probes, Inc., of Eugene, Oregon, sets forth additional fluorescent dyes that may be used in constructing the double-labeled substrates of the present invention. However, the dyes listed here, as well as those described within in the *Handbook of Fluorescent Probes and Research Chemicals*, are provided for illustrative purposes only and do not comprise a comprehensive list of the dyes usable in the context of the present invention.

A double-labeled substrate according to the present invention may include a non-fluorescent dye and a fluorescent dye, or, alternatively, a double labeled substrate according to the present invention may be constructed using two fluorescent dyes. However, it should be noted that the structure of the GFP and related protein molecules might not be able to stack and quench in the same manner as conventional dyes. As a result, where double-labeled substrates according to the present invention are constructed using one of the various GFP molecules, it may be necessary to include only one GFP molecule in the combination of two dyes covalently attached to the double-labeled substrate (*See*, U.S. Patents 5,958,713, 5,925,558, and 5,912,137). Nevertheless, the combination, nature and location of the two dyes included in a

double-labeled substrate of the present invention is of relatively little import, provided that the two dyes stack to form a quenched intramolecular dye dimer when the double-labeled substrate is unmodified, the two dyes dissociate upon structural modification of the double-labeled substrate, and the dissociation of the two dyes upon structural modification results in a change in the double-labeled substrate's fluorescence or absorbance characteristics. Thus, the construction of a double-labeled substrate according to the present invention is variable, and, depending on the application, the double-labeled substrate of the present invention may include one or more of many biomolecular substrates and any suitable combination of two labels.

Also included within the scope of the present invention are methods of using the double- labeled biomolecular substrates of the present invention. For example, methods of using the double-labeled substrates herein described for the assay of protein kinase activity *in vitro* and in living cells fall within the scope of the present invention, as do methods of using the same double-labeled substrates for high-throughput screening. The double-labeled substrates are also useful for diagnostic and therapeutic applications and for methods which facilitate the discovery of substrates, activators, and inhibitors for novel protein kinases. Significantly, most efforts to discover drugs affecting protein kinase activity are presently aimed at screening for possible protein kinase activators and inhibitors.

A preferred method according to the present invention for assaying structural modification of biomolecules *in vitro* is homogenous, comparatively simple, and includes the steps of providing a double-labeled substrate as herein described, including the double-labeled substrate in a sample, and quantifying any resultant change in fluorescence or absorbance resulting from the structural modification of the double-labeled substrate. Because only double-labeled substrate which is structurally modified exhibits a change in fluorescence or absorbance, this method requires no separation of the unmodified double-labeled substrate from the modified double-labeled substrate before the modification of the double-labeled substrate can be accurately assayed. Further, the assay methods of the present invention require no special reagents other than the double-labeled substrate, and the measurement of changes in fluorescence or absorbance of the double-labeled substrate can be easily achieved using a variety of well known instruments, such as, for example, known spectrometers, 96-well and 384-

well microtiter plate readers, other multichannel readers, and micro-array instruments. Therefore, the methods of assaying covalent biomolecular modifications according to the present invention provide advantages over currently used assays in terms of simplicity, throughput, versatility, and economy.

Because the method already described requires no separation steps, it can be easily modified in order to assay processes of covalent biomolecular modification in living cells. A preferred method for assaying biomolecular structural modification in living cells includes providing a double-labeled substrate of the present invention, introducing the double-labeled substrate into living cells using techniques well known in the art, such as microinjection, pinocytosis, or facilitated uptake, and quantifying any change in fluorescence or absorbance resulting from the structural modification of the double-labeled substrate using well known instruments, such as, for example, known spectrometers, fluorescence microscopes, plate readers, cell counters, and cell sorters. Again, this method requires no separation steps (although they may be used), and, thus, allows for the continuous, real-time assay of the processes resulting in structural modification of biomolecules in living cells. Monitoring of biomolecular structural modification activities in living cells could be used for purposes of basic research, drug discovery, diagnosis of disease states, or efficacy of therapy following targeted drug treatment.

Also included within the scope of the invention are methods for the assay of covalent biomolecular modifications performed *in vitro* and in living cells which simultaneously monitor different processes of biomolecular structural modification by utilizing various double-labeled substrates of the present invention, each double-labeled substrate being designed to specifically assay the activity of a different process by which biomolecules are modified. Such a method is similar to those already detailed, except that, in order to accurately simultaneously monitor the activity of multiple processes of covalent biomolecular modification, each of the different double-labeled substrates must be designed with unique and distinguishable spectral properties. Because they enable the simultaneous and continuous monitoring of multiple processes by which biomolecules are structurally modified, such assay methods will likely hasten the discovery of exactly which processes of biomolecular structural modification are associated with specific disease states.

The double-labeled substrates of the present invention can also be used in methods facilitating the discovery of drugs which target intracellular processes of covalent biomolecular modification. Such a preferred method would include the steps of providing a sample containing the modifying enzyme(s) to be targeted, introducing into the sample a drug designed to target a particular intracellular process of covalent biomolecular modification, introducing into the sample a double-labeled substrate specific for the targeted modification process, and quantifying any change in fluorescence or absorbance resulting from the structural modification of the double-labeled substrate using well known instruments. Since the covalent modification of biomolecules represents one of the major mechanisms by which intracellular signaling occurs, many processes facilitating such modifications are likely to be important drug targets, and methods of drug discovery facilitated by the double-labeled peptides of the present invention are of particular importance.

Yet another aspect of the present invention is a method of using the double-labeled substrates of the present invention to identify substrates for novel modifying enzymes. The human genome is estimated to contain thousands of different enzymes responsible for the intracellular modification of biomolecules, many of which are likely to be critically involved in disease processes. Protein kinases represent but one superfamily of such enzymes, yet there are currently hundreds of putative protein kinases in sequence databases, such as GenBank and "EST" databases, whose function and regulation are entirely unknown. These putative protein kinases can be identified by their homology to known protein kinases and can be cloned and expressed as proteins, but their enzymatic properties cannot be studied without an appropriate peptide or protein substrate. A preferred embodiment of a method of identifying peptide substrates for these protein kinases or other putative enzymes with unknown enzymatic properties involves constructing (*e.g.*, synthesizing) combinatorial libraries of double-labeled substrates according to the present invention with core molecular backbones constructed with randomized amino acid (in the case of peptides) and nucleotide (in the case of DNA and RNA, etc.) sequences, systematically introducing individual double-labeled substrates from the combinatorial libraries into a sample containing the novel protein kinase or other enzyme of unknown activity, and quantifying any change in substrate fluorescence or absorbance which results from

covalent modification of the double-labeled substrate. Structural modification of specific double-labeled substrates in the combinatorial library by the novel enzyme of interest would result in a change in their spectral properties, which would permit these biomolecules to be identified by standard methods. Once substrates are identified for a novel enzyme, they can be used to characterize the activity and properties of the enzyme *in vitro* and in living cells. Further, such substrates would also facilitate drug development for the novel enzyme of interest using the methods described herein (*e.g.*, to identify specific inhibitors or activators of a newly discovered protein kinase).

The present invention is further directed to kits which utilize the double-labeled substrates and methods described herein to detect and/or quantify covalent biomolecular modification. A preferred embodiment of such a kit would include a container, one or more different double-labeled substrates of the present invention contained within the container, and instructions for use. The kits may also include, for convenience, buffers and other reagents necessary to carry out the assay, and samples of enzyme for calibration purposes. The reagents included with the kits can be varied depending on the application and in order to optimize the sensitivity of the assay.

A further aspect of the invention is the use of double-labeled substrates to detect protein kinase activities and other modification reactions in living cells. A preferred embodiment of a method of detecting protein kinase activity in living cells involves constructing double-labeled protein kinase substrates as probes which are sufficiently cell permeable, or capable of cell permeability with inducement measures well known to one skilled in the art, and according to the present invention, with core molecular backbones constructed of various peptide sequences. In this situation, the protein kinase activity would result in a covalent structural modification of the double-labeled substrate, leading to a change in fluorescence or absorbance and *in situ* detection of kinase activity using instruments well known in the art, such as, for example, known spectrometers, fluorescence microscopes, plate readers, cell counters, and cell sorters.

The invention is further described with the aid of the following illustrative example.

Example I

A double-labeled protein kinase substrate can be designed, synthesized, characterized, and used to assay the activity of PKA and other protein kinases.

The core molecular backbone of the substrate is the synthetic peptide sequence Asp-Ser-Gln-Arg-Arg-Arg-Glu-Ile-Leu-Ser-Arg-Arg-Pro-Ser-Tyr-Arg-Arg-Ile-Leu-Asn-Asp-Leu-Cys-Gly (SEQ. ID. NO.: 5). This synthetic peptide sequence is based on the native sequence for KID, which is Arg-Arg-Pro-Ser-Tyr-Arg-Lys-Ile-Leu-Asn-Asp-Leu (SEQ. ID. NO.: 6). To arrive at the peptide sequence of the core molecular backbone, the Lys residue of the native KID sequence was replaced by an Arg residue to facilitate site specific labeling of the peptide's α-amino group. Replacing the Lys residue of the native KID sequence resulted in the synthetic KID sequence Arg-Arg-Pro-Ser-Tyr-Arg-Arg-Ile-Leu-Asn-Asp-Leu (SEQ. ID. NO.: 7), which represents the sequence generally referred to herein as "the KID sequence." Next, a Cys residue was added to the C-terminus of the synthetic KID sequence to allow labeling of the molecular backbone with a dye through the sulfhydryl group in the cysteine residue, and a Gly residue was added at the terminal Cys residue to facilitate peptide synthesis. Finally, the additional peptide sequence Asp-Ser-Gln-Arg-Arg-Arg-Glu-Ile-Leu-Ser (SEQ. ID. NO.: 8) was added at the Arg residue of the N-terminus of the KID sequence to give the final peptide more helical structure.

The synthetic peptide sequence of the core molecular backbone of the substrate was synthesized on a benzhydrylamine resin using conventional (tBOC) solid phase peptide synthetic chemistry. *See, e.g.* Barany and Merrifield in *The Peptides, Analysis, Synthesis, Biology*, Vol. 2, E. Gross and J. Meienhofer, eds., (Acad. Press, New York, 1980), Glass. D.B., *Methods Enzymol.*, 99, 119-139 (1983). After synthesis, the peptide sequence was cleaved from the resin with anhydrous HF using standard protocols which yield a crude side-chain deprotected peptide with an amide C-terminus, and the synthetic peptide sequence was purified to homogeneity by HPLC using a C4 reverse-phase column. The mass of the purified peptide sequence was confirmed by mass spectrometry. The synthetic polypeptide sequence of SEQ. ID. NO.: 5 was selected as an exemplary core molecular backbone for a double-labeled substrate of the present invention not only because the KID sequence embedded in the synthetic polypeptide is known to contain the specificity determinants of several protein kinases, including

PKA, but also because this exemplary synthetic peptide sequence has proven to undergo a phosphorylation-dependent change in conformation.

After purification, the core molecular backbone was conjugated with two dyes to form a double-labeled substrate. First, the synthetic peptide sequence of the core molecular backbone was conjugated with tetramethylrhodamine-5-maleimide. The maleimide on the dye reacts with the cysteine residue at the C-terminus in the KID region, and the maleimide group serves as the link between the sulfhydryl group on the cysteine and the rhodamine group. The single-labeled substrate was then conjugated at the N-terminus with either 5-carboxyfluorescein, succinimidyl ester or 5-carboxytetramethylrhodamine, succinimidyl ester. The succinimidyl ester group reacts with the amino group at the N-terminus of each peptide to form a carboxamide bond with the dye. Following HPLC purification on a C4 reversed-phase column, the double-labeled substrate was subjected to analysis by mass spectrometry analysis, UV absorbance spectrophotometry, and fluorescence spectrophotometry.

After such analyses, the double-labeled substrate was phosphorylated with PKA. The phosphate acceptor amino acid in this double-labeled substrate is the serine residue found within the embedded KID sequence.

As illustrated in FIG. 5 through FIG. 9, phosphorylation of double-labeled substrates prepared as herein described results in detectable changes in the absorbance and fluorescence characteristics of the dyes included in the double-labeled substrates.

FIG. 5. illustrates the absorbance peaks for fluorescein and rhodamine before and after phosphorylation of a double-labeled substrate having a fluorescein dye and a rhodamine dye molecule conjugated thereto. The unphosphorylated substrate exhibits an absorbance maximum for fluorescein at 500nm and an absorbance maximum for rhodamine at 552 nm. However, after phosphorylation, the two absorbance peaks shift to 498 nm and 548 nm for fluorescein and rhodamine, respectively.

FIG. 6 and 7 illustrate the even more dramatic phosphorylation dependent changes in the fluorescence characteristics a double-labeled substrate having a fluorescein and a rhodamine molecule conjugated thereto. As can be seen in FIG. 6, the fluorescence of the fluorescein label increased 340% after phosphorylation, and FIG. 7 illustrates that phosphorylation of the double-labeled substrate caused a 35% increase in the fluorescence of the rhodamine label.

As mentioned, the molecular backbone described herein may also be labeled with, among other combinations, two rhodamine dyes instead of a rhodamine dye and a fluorescein dye, and FIG. 8 and FIG. 9 illustrate the phosphorylation-dependent changes in the optical properties of such a double-labeled substrate. As can be seen in FIG. 8, in its unphosphorylated state, the double-labeled substrate exhibited two absorbance maxima. The larger peak is at 520 nm, while the smaller peak is at 552 nm. After phosphorylation, the peak at 520 nm decreases in size while the peak at 552 nm shifts to 550 nm and increases in size. Moreover, as can be appreciated from FIG. 9, the fluorescence of rhodamine increases 69% after phosphorylation.

The results illustrated in FIG. 5 through FIG. 9, therefore, show that the fluorescence of at least one label conjugated to the double-labeled substrates is quenched when the substrates are found in their unphosphorylated state. These results indicate that when the double-labeled substrates are not phosphorylated, the two dyes included in each double-labeled substrate stack on each other to form an intramolecular dye dimer, resulting in the reduction of the fluorescence of at least one of the dyes included in the dye dimers. Stacking is also indicated by the observation that the UV absorbance spectrum of the unphosphorylated double-labeled substrate differs markedly from the spectra of the same substrate after phosphorylation.

As is also apparent by reference to results illustrated in FIG. 5 through FIG. 9, phosphorylation of the double-labeled substrates results in an increase in the intensity of the fluorescent emission peak of at least one dye conjugated to the double-labeled substrates. This indicates that phosphorylation of the double-labeled substrate causes a dissociation of the intramolecular dimer. In each instance, there was a large increase in fluorescence intensity of at least one of the dyes conjugated to the double-labeled substrate, thereby providing a high signal-to-noise ratio. Sensitivity is also excellent with changes in dye emission intensity being observable at low nanomolar concentrations of peptide in a standard spectrofluorometer. The favorable sensitivity and signal-to-noise ratio indicate the double-labeled substrate will be useful for monitoring protein kinase activity in a variety of applications.

The procedures and methods described herein can be employed to prepare and use double-labeled protein kinase substrates for assaying most any other protein kinase. For example, the KID sequence included in the core molecular backbone described

herein may be modified to contain an appropriate consensus sequence for a given protein kinase determinant. Such consensus sequences can be found in the literature for many common kinases such as PKA, PKC, CaM kinase II, etc. (*cf.*, Songyang et al. Current Biol. 4:479, 1994). Moreover, the double-labeled substrate of the present invention can be prepared for assaying most any other intracellular processes leading to the structural modification of protein or other biomolecules. Thus, even though the present invention has been herein described in terms of certain preferred embodiments and specific examples, such descriptions are illustrative only and do not limit the scope of the present invention. The scope of the present invention is to be defined by the appended claims.

CLAIMS

What is claimed is:

1. A biomolecular substrate comprising:

a core molecular backbone;

a first fluorescent dye associated with said core molecular backbone; and

a second dye associated with said core molecular backbone which, when said biomolecular substrate is not covalently modified, associates with said first fluorescent dye forming a quenched intramolecular dye dimer, but which, when said biomolecular substrate is covalently modified, dissociates from said first fluorescent dye .

2. The biomolecular substrate of claim 1, wherein said second dye is a fluorescent dye.

3. The biomolecular substrate of claim 1 further comprising a first spacer segment included at a first terminus of said core molecular backbone.

4. The biomolecular substrate of claim 1 further comprising a first spacer segment included at a first terminus of said core molecular backbone and a second spacer segment included at a second terminus of said core molecular backbone.

5. The biomolecular substrate of claim 1, wherein said first fluorescent dye is selected from a group consisting of fluorescein, rhodamine, cyanine, Oregon Green, Texas Red, Lucifer Yellow, BODIPY, rhodol, coumarin, pyrene, eosin, erythrosin, napthalene, pyridyloxazole, anthrancene, fluorescamine, acridine, benzofuran, anthranilic acid, aminobenzoic acid, N-methylisatoic acid, isoluminol, bezoxadiazole, carboxybenzoyl-quinoline-carboxyaldehyde, salicylate, bimane, phenathroline, Yellow Fluorescent Protein, and Green Fluorescent Protein.

6. The biomolecular substrate of claim 1, wherein said second dye is selected from a group consisting of fluorescein, rhodamine, cyanine, Oregon Green, Texas Red, Lucifer Yellow, BODIPY, rhodol, coumarin, pyrene, eosin, erythrosin, napthalene, pyridyloxazole, anthrancene, fluorescamine, acridine, benzofuran.

anthranilic acid, aminobenzoic acid, N-methylisatoic acid, isoluminol, bezoxadiazole, carboxybenzoyl-quinoline-carboxyaldehyde, salicylate, bimane, phenathroline, Yellow Fluorescent Protein, and Green Fluorescent Protein.

7. The biomolecular substrate of claim 1, wherein said core molecular backbone comprises a molecule selected from a group consisting of a peptide, a protein, a nucleic acid, a sugar, a lipid, a receptor, and a biopolymer.

8. The biomolecular substrate of claim 1, wherein said core molecular backbone comprises an amino acid sequence.

9. The biomolecular substrate of claim 1, wherein said core molecular backbone includes a substrate determinant.

10. The biomolecular substrate of claim 1, wherein said core molecular backbone includes a protein kinase substrate.

11. The biomolecular substrate of claim 1, wherein said core molecular backbone includes a nucleotide sequence.

12. The biomolecular substrate of claim 1, wherein said core molecular backbone includes a lipid.

13. The biomolecular substrate of claim 1, wherein said core molecular backbone includes a biopolymer comprising a covalent combination of molecules selected from the group consisting of amino acids, nucleic acids, sugars, and lipids.

14. The protein kinase substrate of claim 1, wherein the core molecular backbone comprises a KID peptide sequence, the first fluorescent dye comprises fluorescein-succinimidyl ester, and the second dye comprises tetramethylrhodamine-maleimide.

15. A method of assaying covalent biomolecular modification in a sample comprising:

providing the sample with a biomolecular substrate comprising:

a biomolecular substrate;

a core molecular backbone;

a first fluorescent dye associated with said core molecular backbone; and

a second dye associated with said core molecular backbone which, when said biomolecular substrate is not covalently modified, associates with said first fluorescent dye forming a quenched intramolecular dye dimer and affecting fluorescence or absorbance characteristics of said biomolecular substrate, but which dissociates from said first fluorescent dye when said biomolecular substrate is covalently modified;

introducing said biomolecular substrate to said sample; and

quantifying a resultant change in said fluorescence or absorbance characteristics of said biomolecular substrate.

16. The method of claim 15, wherein said biomolecular substrate is introduced into said living cells.

17. The method of claim 15, wherein said sample includes a drug targeting a specific process of covalent biomolecular modification.

18. The method of claim 16, wherein said sample includes a drug targeting a specific process of covalent biomolecular modification.

19. The method of claims 15, wherein said step of providing a biomolecular substrate comprises providing two or more different biomolecular substrates, each of said two or more biomolecular substrates being specific for different processes of covalent biomolecular modification and having unique and distinguishable spectral properties.

20. The method of claim 16, wherein said step of providing a biomolecular substrate comprises providing two or more different biomolecular substrates, each of said two or more biomolecular substrates being specific for different processes of covalent biomolecular modification and having unique and distinguishable spectral properties.

21. The method of claim 15, wherein the step of quantifying the resultant change in said fluorescence or absorbance characteristics of said biomolecular substrate comprises quantifying the resultant change in fluorescence or absorbance of said biomolecular substrate without separating biomolecular substrate which has been covalently modified from biomolecular substrate which has not been covalently modified.

22. The method of claim 16, wherein the step of quantifying the resultant change in fluorescence or absorbance characteristics of said biomolecular substrate comprises quantifying the resultant change in fluorescence or absorbance of said biomolecular substrate without separating biomolecular substrate which has been covalently modified from biomolecular substrate which has not been covalently modified.

23. A method of assaying protein kinase activity comprising:

providing a biomolecular substrate comprising:

a KID peptide sequence;

a molecule of fluorescein; and

a molecule of tetramethylrhodamine which, when said biomolecular substrate is not phosphorylated, associates with said molecule of fluorescein forming an intramolecular dye dimer, but which dissociates from said molecule of fluorescein when said biomolecular substrate is phosphorylated by a protein kinase;

providing a sample;

introducing said protein kinase substrate to said sample; and

quantifying a resultant change in fluorescence or absorbance of said biomolecular substrate.

24. The method of claim 23, wherein the step of quantifying the resultant change in fluorescence or absorbance of said biomolecular substrate comprises quantifying the resultant change in fluorescence or absorbance of said biomolecular substrate without separating biomolecular substrate which has been phosphorylated from biomolecular substrate which has not been phosphorylated.

25. A method of identifying substrates of novel enzymes which catalyze covalent structural modifications of particular proteins or peptide sequences comprising:

gathering a combinatorial library of unique double-labeled substrates, said unique double-labeled substrates each comprising:

a particular, randomized core amino acid sequence;

a first fluorescent dye associated with said particular, randomized core amino acid sequence; and

a second dye associated with said particular, randomized core amino acid sequence which, when said unique double-labeled substrates are not covalently modified, associates with said first fluorescent dye forming a quenched intramolecular dye dimer and affecting the fluorescence or absorbance characteristics of said unique double-labeled substrates, but which dissociates from said first fluorescent dye when said unique double-labeled substrates are covalently modified;

systematically contacting each of said unique double-labeled substrates with a novel enzyme;

quantifying any change in fluorescence or absorbance characteristics of each of said unique double-labeled substrates;

selecting members of the library undergoing a fluorescence change or an absorbance change; and

determining the amino acid sequence of said selected members of the library.

26. A kit comprising:

a container;

one or more different biomolecular substrates contained within said container, each of said one or more different biomolecular substrates comprising:

a core molecular backbone;

a first fluorescent dye associated with said core molecular backbone;

a second dye associated with said core molecular backbone which, when said biomolecular substrate is not covalently modified, associates with said first fluorescent dye forming an intramolecular dye dimer, but which dissociates from said first fluorescent dye when said biomolecular substrate is catalytically or non-catalytically covalently modified; and

a sample of enzyme standard with which to standardize the assay.

27. A method of identifying substrates of novel enzymes which catalyze covalent structural modifications of particular nucleic acids comprising:

gathering a combinatorial library of unique double-labeled substrates, said unique double-labeled substrates each comprising:

a particular, randomized core nucleic acid sequence;

a first fluorescent dye associated with said particular, randomized core nucleic acid sequence; and

a second dye associated with said particular, randomized core nucleic acid sequence which, when said unique double-labeled substrates are not covalently modified, associates with said first fluorescent dye forming a quenched intramolecular dye dimer and affecting the fluorescence or absorbance characteristics of said unique double-labeled substrates, but which dissociates from said first fluorescent dye when said unique double-labeled substrates are covalently modified;

systematically contacting each of said unique double-labeled substrates with a novel enzyme;

quantifying any change in fluorescence or absorbance of each of said unique double-labeled substrates;

selecting members of the library undergoing a fluorescence or absorbance change; and

determining the nucleotide sequence of said selected members of the library.

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(54) Title: HOMOGENEOUS FLUORESCENCE METHOD FOR ASSAYING STRUCTURAL MODIFICATIONS OF BIOMOLECULES

(57) Abstract: Double-labeled protein biomolecular substrates and methods for the homogenous assay of processes by which biomolecules are covalently modified are described. The methods of the present invention utilize biomolecular substrates labeled at two positions with two fluorescent dyes or with a fluorescent dye and a nonfluorescent dye. The two labeling dyes of the unmodified biomolecular substrates stack, thereby quenching the substrate's fluorescence. Upon covalent modification of the double-labeled substrate, however, the intramolecularly stacked dyes dissociate and the fluorescence of the phosphorylated substrate changes markedly. Methods utilizing the double-labeled substrates of the present invention do not require physical separation of modified and unmodified substrate molecules, nor do they require other special reagents or radioactive materials. Methods for preparing and characterizing the substrates used in the assay procedure are described, as are methods utilizing the substrates of the present invention for high-throughput screening, for monitoring intracellular processes of covalent biomolecular modification in living cells, for diagnostic and therapeutic applications for diseases involving dysfunctional processes of covalent biomolecular modification, and for discovering novel enzymatic substrates.

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FIG. 1

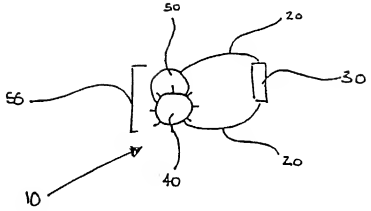


FIG. 2

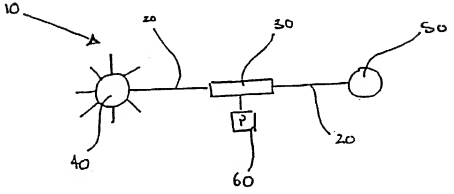


FIG. 3

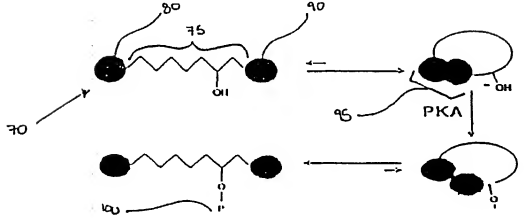


FIG. 4

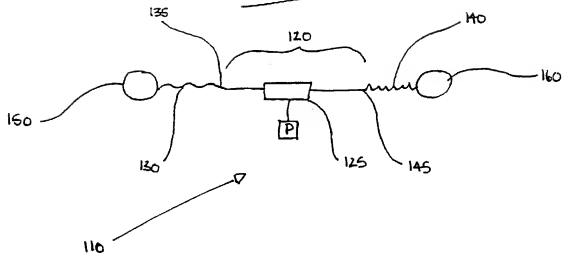
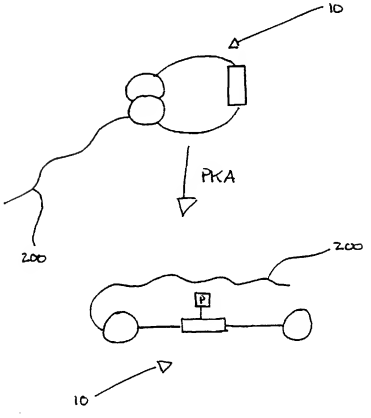


FIG. 10



Absorbance Spectrum of F-DSQRRREILS-KID-R After PKA Phosphorylation

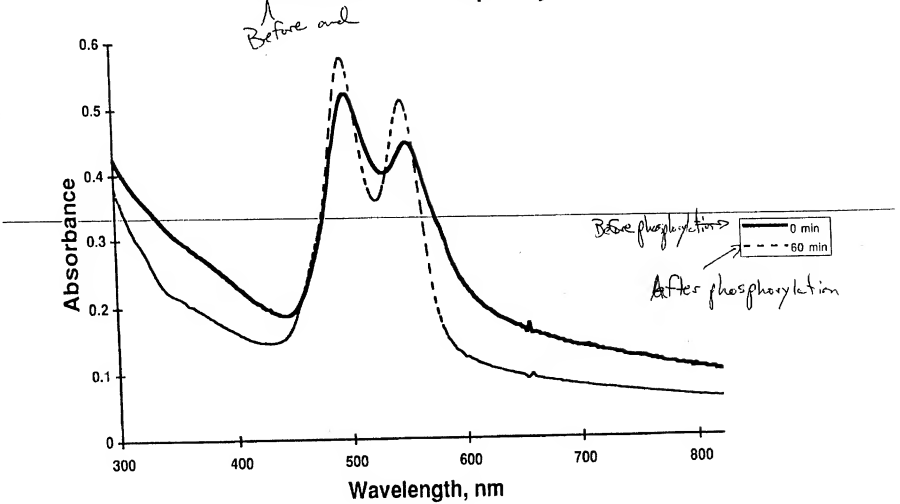


FIG. 5

Ex 480 nm Chart 1
F-DSQRRREILS-KID-R Fluorescence, After
PKA Phosphorylation, Ex 480 nm (Fluorescein)
Before and
0 min
-60 min
Before
After
Fluorescence
Wavelength, nm
0
2000
4000
6000
8000
10000
12000
14000
16000
18000
490
500
510
520
530
540
550

FIG. 6

F-DSQRRREILS-KID-R Fluorescence/After PKA
Phosphorylation, Ex 535 nm → (Rhodamine)
Before
After
0 min
~60 min
Fluorescence
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
545
555
565
575
585
595
605
615
625
635
645
Wavelength, nm

FIG. 7

Fig. 4

Absorbance Spectrum of TMR-DSQRRREILS-KID-TMR After PKA Phosphorylation
Before and
0 min
120 min
Before
After
Absorbance
5.00E-01
4.00E-01
3.00E-01
2.00E-01
1.00E-01
0.00E+00
-1.00E-01
300
400
500
600
700
800
Wavelength, nm

FIG. 8

Fluorescence of TMR-DSQRRREILS-KID-TMR
After PKA Phosphorylation, Ex 545 nm
Before and
0 min
100 min
Before
After
Fluorescence
12000
10000
8000
6000
4000
2000
0
555
565
575
585
595
605
615
625
635
645
655
Wavelength, nm

FIG. 9

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Donald K. Blumenthal II	**Examiner:**	Not Yet Assigned
Serial No.:	10/048,244	**Group Art Unit:**	Not Yet Assigned
Filed:	January 25, 2002	**Attorney Docket No.:**	0274-3858.1US
Title:	HOMOGENEOUS FLUORESCENCE METHOD FOR ASSAYING STRUCTURAL MODIFICATIONS OF BIOMOLECULES		

POWER OF ATTORNEY BY ASSIGNEE
AND CERTIFICATE UNDER 37 CFR § 3.73(b)

Commissioner for Patents
Washington, D.C. 20231

Sir:

University of Utah, assignee of the entire right, title and interest by assignment from the inventor(s) in the above-identified application, hereby appoints the following attorneys and agents:

David V. Trask, Reg. No. 22,012	William S. Britt, Reg. No. 20,969	Laurence B. Bond, Reg. No. 30,549
Joseph A. Walkowski, Reg. No. 28,765	James R. Duzan, Reg. No. 28,393	H. Dickson Burton, Reg. No. 48,396
Allen C. Turner, Reg. No. 33,041	Edgar R. Cataxinos, Reg. No. 39,931	Kent S. Burningham, Reg. No. 30,453
Brick G. Power, Reg. No. 38,581	Kevin K. Johanson, Reg. No. 38,506	Paul C. Oestreich, Reg. No. 44,983
Devin R. Jensen, Reg. No. 44,805	Krista Weber Powell, Reg. No. 47,867	Shawn G. Hansen, Reg. No. 42,627
Bretton L. Crockett, Reg. No. 44,632	Tawni L. Wilhelm, Reg. No. 47,456	Bradley B. Jensen, Reg. No. 46,801
Andrew F. Nilles, Reg. No. 47,825	Greg T. Warder, Reg. No. 50,208	Katherine A. Hamer, Reg. No. 47,628
Mardson Q. McQuay, Reg. No. P-52,020	Marcus S. Simon, Reg. No. 50,258	Jeff M. Michelsen, Reg. No. 50,978
Trent N. Butcher, Reg. No. P-51,518	G. Scott Dorland, Reg. No. P-51,622	

as its attorneys with full power of substitution to prosecute this application and all applications claiming filing date priority therefrom and to transact all business in the U.S. Patent and Trademark Office in connection therewith.

The above-identified assignee hereby elects, pursuant to 37 C.F.R. § 3.71, to conduct the prosecution of the above-identified patent application to the exclusion of the inventor(s).

A chain of title from the inventor(s) of the above-identified patent application to the above-identified assignee is shown:

☐ In an assignment recorded in the U.S. Patent and Trademark Office at Reel _____, Frame _____.

☒ In an assignment filed herewith for recordation, a true copy of which is attached hereto.

The undersigned has reviewed the above-identified assignment and, to the best of his knowledge and belief, title is in the above-identified assignee.

The undersigned further avers that he is empowered to make and sign the foregoing certification on behalf of the above-identified assignee, and to take the action set forth herein on its behalf.

Please direct all communications regarding the above-identified application to:

Laurence B. Bond,
TRASKBRITT
P.O. Box 2550
Salt Lake City, UT 84110
Telephone: (801) 532-1922
Fax: (801) 531-9168

Respectfully submitted,

University of Utah

Date: 08-16-02

By: RKKulkarni

Name: Rajiv K. Kulkarni, PhD, MBA

Its: Senior Licensing Manager

Document in ProLaw

THE UNIVERSITY OF UTAH

September 17, 2001

TO: Dr. Raymond Gesteland

FROM: Chris Jansen, Ph.D., Director CJ

RE: Delegation of Signatory Authority

As routine matters are performed in our office in the name of the University. I am requesting that you delegate authority to Rajiv Kulkarni to execute documents on behalf of the University and the University of Utah Research Foundation for Patent Documents, PCT Authorizations, Confidential Disclosure Agreements, and Biological and Materials Transfer Agreements.

Approved: RF Gesteland

Raymond Gesteland
Vice President for Research/
President, University of
Utah Research Foundation

Technology Transfer Office
615 Arapeen Drive, Suite 110
Salt Lake City, Utah 84108
(801) 581-7792
FAX: (801) 581-7538

Attorney Docket No. 0274-3858.1US

ASSIGNMENT

In consideration of One Dollar ($1.00), and other good and valuable consideration including such remuneration as provided for by the University of Utah's patent policy 6-4 regarding sharing of revenues from licensing, the receipt of which is hereby acknowledged, each undersigned ASSIGNOR does hereby:

SELL, ASSIGN AND TRANSFER the to the University of Utah, ("ASSIGNEE"), a non-profit organization existing under the laws of the state of Utah, entire right, title and interest for the United States and all foreign countries in and to any and all improvements which are disclosed in the Provisional Application for United States Letters Patent Serial No. 60/145,755 filed on July 27, 1999, and entitled **HOMOGENEOUS FLUORESCENCE METHOD FOR ASSAYING STRUCTURAL MODIFICATIONS OF BIOMOLECULES**, in the PCT International Application no. PCT/US00/40495 filed on July 27, 2000 entitled **HOMOGENEOUS FLUORESCENCE METHOD FOR ASSAYING STRUCTURAL MODIFICATIONS OF BIOMOLECULES**, and in the Application for United States Letters Patent Serial No. 10/048,244 filed January 25, 2002 entitled **HOMOGENEOUS FLUORESCENCE METHOD FOR ASSAYING STRUCTURAL MODIFICATIONS OF BIOMOLECULES**, such applications and all divisional, continuing, substitute, renewal, reissue and all other applications for patent or the legal equivalent thereof which have been or may be filed in the United States and all foreign countries relating to any of such improvements; all original, reexamined and reissued patents which have been or shall be issued in the United States and all foreign countries on such improvements; and specifically including the right to file foreign applications under the provisions of any convention or treaty and claim priority based on such application made in the United States;

AUTHORIZE the ASSIGNEE to apply for and receive any and all United States and foreign patents relating to such improvements in its own name;

AUTHORIZE AND REQUEST the issuing authority to issue any and all United States and foreign patents granted on such improvements to and in the name of the ASSIGNEE;

WARRANT AND COVENANT that no assignment, grant, mortgage, license or other agreement or encumbrance affecting the rights and property herein conveyed has been or will be made or entered into by the undersigned, and that the full right to convey the same as herein expressed is possessed by the undersigned;

COVENANT, when requested and at the expense of the ASSIGNEE, to carry out in good faith the intent and purpose of this assignment, to execute all divisional, continuing, substitute, renewal, reissue, and all other patent applications relating to any and all such improvements; to execute all rightful oaths, declarations, assignments, powers of attorney and other papers; to communicate to the ASSIGNEE all facts and provide to the ASSIGNEE all documents and things known and accessible to the undersigned relating to such improvements and the history thereof, and testify as to the same in any interference, litigation or other proceeding relating thereto; and generally to do everything possible which the ASSIGNEE shall consider desirable for vesting title to such improvements in the ASSIGNEE, and to secure, maintain, defend and enforce valid and enforceable patent protection for such improvements;

AGREE AND ACKNOWLEDGE that the SALE, ASSIGNMENT AND TRANSFER of rights and property set forth herein is and shall be IRREVOCABLE and BINDING upon the heirs, assigns, representatives and successors of each undersigned ASSIGNOR and EXTEND to the successors, assigns and nominees of the ASSIGNEE.

ASSIGNOR:

Donald K. Blumenthal II

Date 8/16/2002

COUNTY OF Salt Lake
: ss.
STATE OF Utah)

On this 16th day of August, 2002, before me personally appeared Donald K. Blumenthal II to me known to be the person whose name is subscribed to the foregoing instrument, and acknowledged to me that he/she executed the same.

Sheri L. Gordon
Notary Public in and for said County and State

My Commission Expires:

June 23, 2004

NOTARY PUBLIC
SHERI L. GORDON
615 Arapeen Dr., Ste. 110
Salt Lake City, Utah 84108
My Commission Expires
June 23, 2004
STATE OF UTAH

Document in ProLaw

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Donald K. Blumenthal II	**Examiner:**	Not Yet Assigned
Serial No.:	10/048,244	**Group Art Unit:**	Not Yet Assigned
Filed:	January 25, 2002	**Attorney Docket No.:**	0274-3858.1US
Title:	HOMOGENEOUS FLUORESCENCE METHOD FOR ASSAYING STRUCTURAL MODIFICATIONS OF BIOMOLECULES		

POWER OF ATTORNEY BY ASSIGNEE
AND CERTIFICATE UNDER 37 CFR § 3.73(b)

Commissioner for Patents
Washington, D.C. 20231

Sir:

University of Utah Research Foundation, assignee of the entire right, title and interest by assignment from the inventor(s) in the above-identified application, hereby appoints the following attorneys and agents:

David V. Trask, Reg. No. 22,012	William S. Britt, Reg. No. 20,969	Laurence B. Bond, Reg. No. 30,549
Joseph A. Walkowski, Reg. No. 28,765	James R. Duzan, Reg. No. 28,393	H. Dickson Burton, Reg. No. 48,396
Allen C. Turner, Reg. No. 33,041	Edgar R. Cataxinos, Reg. No. 39,931	Kent S. Burningham, Reg. No. 30,453
Brick G. Power, Reg. No. 38,581	Kevin K. Johanson, Reg. No. 38,506	Paul C. Oestreich, Reg. No. 44,983
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Trent N. Buteber, Reg. No. P-51,518	G. Scott Dorland, Reg. No. P-51,622	

as its attorneys with full power of substitution to prosecute this application and all applications claiming filing date priority therefrom and to transact all business in the U.S. Patent and Trademark Office in connection therewith.

The above-identified assignee hereby elects, pursuant to 37 C.F.R. § 3.71, to conduct the prosecution of the above-identified patent application to the exclusion of the inventor(s).

A chain of title from the inventor(s) of the above-identified patent application to the above-identified assignee is shown:

☐ In an assignment recorded in the U.S. Patent and Trademark Office at Reel _____, Frame _____.

☒ In an assignment filed herewith for recordation, a true copy of which is attached hereto.

The undersigned has reviewed the above-identified assignment and, to the best of his knowledge and belief, title is in the above-identified assignee.

The undersigned further avers that he is empowered to make and sign the foregoing certification on behalf of the above-identified assignee, and to take the action set forth herein on its behalf.

Please direct all communications regarding the above-identified application to:

Laurence B. Bond,
TRASKBRITT
P.O. Box 2550
Salt Lake City, UT 84110
Telephone: (801) 532-1922
Fax: (801) 531-9168

Respectfully submitted,

University of Utah Research Foundation

Date: 8-16-02

By: RKKulkarni

Name: Rajiv K. Kulkarni, PhD, MBA

Its: Senior Licensing Manager

Document in ProLaw

THE UNIVERSITY OF UTAH

September 17, 2001

TO: Dr. Raymond Gesteland

FROM: Chris Jansen, Ph.D., Director CJ

RE: Delegation of Signatory Authority

As routine matters are performed in our office in the name of the University. I am requesting that you delegate authority to Rajiv Kulkarni to execute documents on behalf of the University and the University of Utah Research Foundation for Patent Documents, PCT Authorizations, Confidential Disclosure Agreements, and Biological and Materials Transfer Agreements.

Approved: R F Gesteland

Raymond Gesteland
Vice President for Research/
President, University of
Utah Research Foundation

Technology Transfer Office
615 Arapeen Drive, Suite 110
Salt Lake City, Utah 84108
(801) 581-7792
FAX: (801) 581-7538

Attorney Docket No. 0274-3858.1US

ASSIGNMENT

In consideration of One Dollar ($1.00), and other good and valuable consideration, the receipt of which is hereby acknowledged, the University of Utah ("ASSIGNOR"), hereby:

Sells, assigns and transfers to the University of Utah Research Foundation, ("ASSIGNEE"), a non-profit organization existing under the laws of the State of Utah, its successors, assigns and legal representatives, the entire right, title and interest for the United States and all foreign countries, including priority rights, in and to any and all improvements for which all improvements which are disclosed in the Provisional Application for United States Letters Patent Serial No. 60/145,755 filed on July 27, 1999, and entitled **HOMOGENEOUS FLUORESCENCE METHOD FOR ASSAYING STRUCTURAL MODIFICATIONS OF BIOMOLECULES**, in the PCT International Application no. PCT/US00/40495 filed on July 27, 2000 entitled **HOMOGENEOUS FLUORESCENCE METHOD FOR ASSAYING STRUCTURAL MODIFICATIONS OF BIOMOLECULES**, and in the Application for United States Letters Patent Serial No. 10/048,244 filed January 25, 2002 entitled **HOMOGENEOUS FLUORESCENCE METHOD FOR ASSAYING STRUCTURAL MODIFICATIONS OF BIOMOLECULES**, and in and to said application and all divisional, continuing, substitute, renewal, reissue, and all other applications for Letters Patent which have been or shall be filed in the United States and all foreign countries on any of said improvements; and in and to all original and reissued patents which have been or shall be issued in the United States and all foreign countries on said improvements;

Agrees that said ASSIGNEE may apply for and receive Letters Patent for said improvements in its own name; and that, when requested, without charge to but at the expense of said ASSIGNEE, its successors, assigns and legal representatives, to carry out in good faith the intent and purpose of this assignment, the undersigned will execute all divisional, continuing, substitute, renewal, reissue, and all other patent applications on any and all said improvements; execute all rightful oaths, assignments, powers of attorney and other papers; communicate to said ASSIGNEE, its successors, assigns, and representatives, all facts known to the undersigned relating to said improvements and the history thereof; and generally do everything possible which said ASSIGNEE, its successors, assigns or representatives shall consider desirable for aiding in

securing and maintaining proper patent protection for said improvements and for vesting title to said improvements and all applications for patents and all patents on said improvements, in said ASSIGNEE, its successors, assigns and legal representatives; and

Covenants with said ASSIGNEE, its successors, assigns and legal representatives that no assignment, grant, mortgage, license or other agreement affecting the rights and property herein conveyed has been made to others by the undersigned, and that full right to convey the same as herein expressed is possessed by the undersigned.

ASSIGNOR:
UNIVERSITY OF UTAH

8-16-02
Date

RKKulkarni
Name:
Title: Senior Licensing Manager

STATE OF UTAH)
: ss.
County of Salt Lake)

Before me this 16th day of August, 2002, personally appeared Rajiv Kulkarni, the person who is described in and who executed the above instrument, and acknowledged to me that she executed the same of her own free will for the purpose therein set forth.

Sheri L. Gordon
Notary Public

My Commission Expires

June 23, 2004
Document in ProLaw

NOTARY PUBLIC
SHERI L. GORDON
615 Arapeen Dr., Ste. 110
Salt Lake City, Utah 84108
My Commission Expires
June 23, 2004
STATE OF UTAH

PTO/SB/22 (10-00)
Approved for use through 10/31/2002. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)

Docket Number (Optional) 0274-3858.1US

In re Application of Donald K. Blumenthal II	
Application Number 10/048,244	Filed January 25, 2002
For HOMOGENEOUS FLUORESCENCE METHOD FOR ASSAYING STRUCTURAL MODIFICATIONS OF BIOMOLECULES	
Group Art Unit To Be Assigned	Examiner To Be Assigned

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a response in the above identified application.

The requested extension and appropriate non-small-entity fee are as follows (check time period desired):

- ☐ One month (37 CFR 1.17(a)(1)) $____
- ☐ Two months (37 CFR 1.17(a)(2)) $____
- ☒ Three months (37 CFR 1.17(a)(3)) $920.00
- ☐ Four months (37 CFR 1.17(a)(4)) $____
- ☐ Five months (37 CFR 1.17(a)(5)) $____

☒ Applicant claims small entity status. See 37 CFR 1.27. Therefore, the fee amount shown above is reduced by one-half, and the resulting fee is: $ 460.00.

☒ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The Commissioner has already been authorized to charge fees in this application to a Deposit Account.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 20-1469.

I have enclosed a duplicate copy of this sheet.

I am the ☐ applicant/inventor.

☐ assignee of record of the entire interest. See 37 CFR 3.71

Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96).

☒ attorney or agent of record.

☐ attorney or agent under 37 CFR 1.34(a).

Registration number if acting under 37 CFR 1.34(a).

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

August 27, 2002
Date

Signature

Laurence B. Bond Reg. No. 30,549
Typed or printed name

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

☐ *Total of ____ forms are submitted.

CERTIFICATE OF MAILING

Express Mail Label Number: EV092595277US

Date of Deposit: August 27, 2002

Person Making Deposit: Laurence B. Bond

DECLARATION FOR PATENT APPLICATION (WITH POWER OF ATTORNEY)

As an inventor named below or on any attached continuation page, I hereby declare that:
My residence, post office address and citizenship are as stated next to my name.
I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled HOMOGENEOUS FLUORESCENCE METHOD FOR ASSAYING STRUCTURAL MODIFICATIONS OF BIOMOLECULES, the specification of which (check one):

- ☐ is attached hereto.
- ☐ as filed on _____ as United States application serial no. _____ and was amended on _____.
- ☒ as filed on July 27, 2000 as PCT international application no. PCT/US00/40495 and was amended under PCT Article 19 on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to the patentability of the subject matter claimed in this application, as "materiality" is defined in Title 37, Code of Federal Regulations § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate or § 365(a) of any PCT international application(s) designating at least one country other than the United States of America listed below and on any attached continuation page and have also identified below and on any attached continuation page any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America having a filing date before that of the application(s) on which priority is claimed.

Prior foreign/PCT application(s):

			Priority	Claimed
(number)	(country)	(day/month/year filed)	Yes	No
(number)	(country)	(day/month/year filed)	Yes	No

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or § 365(c) of PCT international application(s) designating the United States of America listed below and on any attached continuation page and, insofar as the subject matter of each of the claims of this application is not disclosed in any such prior application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations § 1.56 which became available

DECLARATION FOR PATENT APPLICATION
(continuation page)

Invention Title: HOMOGENEOUS FLUORESCENCE METHOD FOR ASSAYING STRUCTURAL MODIFICATIONS OF BIOMOLECULES

between the filing date of such prior application and the national or PCT international filing date of this application:

(application serial no.)	(filing date)	(status–pending, patented or abandoned)
(application serial no.)	(filing date)	(status–pending, patented or abandoned)

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below:

60/145,755	July 27, 1999
(provisional application no.)	(filing date)

I hereby appoint the following Registered Practitioners to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

David V. Trask, Reg. No. 22,012
Laurence B. Bond, Reg. No. 30,549
James R. Duzan, Reg. No. 28,393
Allen C. Turner, Reg. No. 33,041
Kent S. Burningham, Reg. No. 30,453
Kevin K. Johanson, Reg. No. 38,506
Devin R. Jensen, Reg. No. 44,805
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Paul C. Oestreich, Reg. No. 44,983
Krista Weber Powell, Reg. No. 47,867
Shawn G. Hansen, Reg. No. 42,627
Tawni L. Wilhelm, Reg. No. 47,456
Andrew F. Nilles, Reg. No. 47,825
Katherine A. Hamer, Reg. No. 47,628

Address all correspondence to: Laurence B. Bond, telephone no. (801) 532-1922.

TRASKBRITT, PC
P.O. Box 2550
Salt Lake City, Utah 84110

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are

Attorney Docket No. 0274-3858.1US

DECLARATION FOR PATENT APPLICATION
(continuation page)

Invention Title: HOMOGENEOUS FLUORESCENCE METHOD FOR ASSAYING STRUCTURAL MODIFICATIONS OF BIOMOLECULES

punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

JDD Full name of first joint inventor: Donald K. Blumenthal II

Inventor's signature _____ Date 8/16/2002

Residence: Salt Lake City, UT

Citizenship: U.S.

Post Office Address: 731 East 17th Avenue, Salt Lake City, UT ~~84112~~ 84103